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Periodontitis in twins : smoking, microbiological and immunological aspects

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Chapter 6

Summary and Discussion

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Periodontitis is a complex, multifactorial, chronic disease that affects the supporting tissues of the teeth and is characterized by inflammation and destruction of connective tissues and alveolar bone (Pihlstrom et al. 2005). Disease expression involves intricate interactions of the bacterial biofilm with the host immune inflammatory response and subsequent alterations in the bone and connective tissue homeostasis (Preshaw 2008). Certain individuals appear to be more susceptible to periodontitis (Loos et al. 2008), and this increased susceptibility is largely determined by the immune inflammatory response that develops in the periodontal tissues following chronic exposure to bacterial plaque (Preshaw 2008). The immune inflammatory response against bacterial plaque can thus be viewed as a 'two-edged sword'. That is, the response is protective by intent, and provides antibodies and polymorphonuclear neutrophils that are responsible for the control of the bacterial infection. However, the inflammatory response in susceptible individuals, results in the local production of excessive quantities of destructive enzymes and inflammatory mediators that result in the tissue destruction which is observed clinically (Preshaw 2008). It is paradoxical that the inflammatory response to the bacterial challenge is primarily responsible for the breakdown of the periodontal hard and soft tissues. Although the bacterial challenge initiates inflammation in the tissues, genetic factors and life style factors, such as smoking, modulate the inflammatory response and determine the resulting disease progression and severity that is seen clinically as bone and attachment loss (Kornman 2008, Loos et al. 2008, Page et al. 1997, Palmer et al. 2005, Pihlstrom et al. 2005, Preshaw 2008).

The present thesis was intended initially to examine the extent to which genetic variation determines phenotypic variation of moderate to severe chronic periodontitis. A better understanding of complex diseases like periodontitis involves the evaluation of the interaction between genetic factors and the life style and bacterial influences. Twin studies have been a valuable source of information about the genetic basis of multifactorial traits. To date results of twin studies of chronic periodontitis show converging results suggesting a substantial role of genetics in this condition (Corey et al. 1993, Michalowicz, 1994, Michalowicz et al. 1991a, Michalowicz et al. 1991b, Michalowicz et al. 2000, Michalowicz et al. 1999, Mucci et al. 2005). However, these

studies have some limitations as they based their results on periodontal observations by questionnaires and not on clinical measurements (Corey et al. 1993, Mucci et al. 2005) and included study populations with relatively minor periodontal destruction (Michalowicz et al. 1991a, Michalowicz et al. 1991b, Michalowicz et al. 2000). Up to now all twin studies selected twins because of their twinship and not on the presence of moderate to severe periodontitis. Thus it may still be questioned to what extent genetics contribute to the development of moderate to severe chronic periodontitis. As prelude to investigating this issue, and motivated by the fact that the interplay between the host genetic make up and smoking as main lifestyle factor is of pivotal importance, it was decided to explore first the effect of smoking on the innate and adaptive immune response in periodontitis. This approach would help us to perhaps explain phenotypic variation among identical twins.

Studies have shown that on average 50% of the patients suffering from periodontitis are smokers or former smokers (Bergstrom 1989, Tonetti 1998, van der Weijden et al. 2001, Xu et al. 2002). This proportion is high compared to the overall adult Dutch population of which approximately 27% are smokers (Tabakspreventie 2008). Most periodontists would consider that smokers are the most challenging patients to manage in the periodontal treatment. Smokers tend to have more advanced periodontal disease than non-smokers and less favorable outcomes following periodontal treatment (Johnson & Hill 2004, Kinane & Chestnutt 2000, Palmer et al. 2005, Tonetti 1998). In addition, smoking has major effects on the host immune response (Graswinckel et al. 2004, Kinane and Chestnutt 2000, Loos et al. 2004, Palmer et al. 2005).

T cells play an important immunoregulatory role in the pathogenesis of periodontal diseases (Gemmell et al. 2007, Kinane & Lappin 2001, Seymour et al. 1996). Evidence supporting the concept that periodontitis is a Th2-type disease comes from both histological analysis of inflamed periodontal tissues and *ex vivo* cytokine production in periodontitis patients compared to healthy controls (Gemmell et al. 2007). However it was not yet investigated whether this Th2 profile in periodontitis would be potentiated by smoking, while at least half of the patients smoke. This presumption set the basis for our first study. In *Chapter 2*, we investigated the monocytic derived T cell directing (Th1/Th2) response and pro-inflammatory cytokine production in *ex vivo* whole blood

cell cultures (WBCC) of smoking and non-smoking chronic periodontal patients. Venous blood was collected from 29 patients (18 non-smokers and 11 smokers) receiving supportive periodontal treatment (SPT), and diluted 10-fold for WBCC. WBCC were stimulated for 18 hours with *Neisseria meningitidis* lipooligosaccharide (LOS) and *Porphyromonas gingivalis* sonic extract (Pg-SE). The production of the T cell directing cytokines interleukin IL-12p40 and IL-10, as well as the pro-inflammatory cytokines IL-1 β , IL-6 and IL-8, was measured in the culture supernatants. Based on previous studies it is clear that monocytes/macrophages are the source for these cytokines (van der Pouw Kraan et al. 1997, van der Pouw Kraan et al. 1995). Our data shows that smoking periodontal patients have a lower IL-12p40/IL-10 ratio after LOS stimulation and lower IL-1 β production after LOS and Pg-SE stimulation than their non-smoking counterparts. These findings were suggestive of a more pronounced Th2 immune response in smoking periodontal patients.

Although our findings provided evidence that smoking patients display a more pronounced Th2 type monocytic response, we felt it needed confirmation that indeed the T cells express such a cytokine pattern. In *Chapter 3*, we investigated the T lymphocytic cytokine production representing Th1 and Th2 subpopulations in smoking and non-smoking patients and healthy controls. WBCC were stimulated with specific T cell stimulants and IFN- γ and IL-13 were measured in the culture supernatants, representing type 1 and 2 Th subpopulations respectively. Our results showed that smokers had more lymphocytes, and higher levels of IFN- γ and IL-13, irrespective of being a periodontal patient. However, in a multivariate analysis we found that the increased IFN- γ production was not significantly explained by smoking, while higher IL-13 was strongly explained by smoking. The secretion of IFN- γ and IL-13 was independent of each other which demonstrate that they indeed were secreted by two distinct T cell populations (i.e. Th1 and Th2 type). We suggested that the increased Th activity and specifically an elevated Th2 profile in smokers may constitute a risk for smoking patients, which may induce conversion of periodontal stability into progressive disease. This study allowed us to confirm our previous finding on the monocytic cytokine profile in smokers. The observed Th2 profile measurable in the monocytic response in the smoking periodontitis patients is consistent with the cytokine profile produced by the lymphocytes.

Our results showed differences between smoking and non-smoking periodontal patients in *ex vivo* cell culture cytokine production. IL-12 and IL-10 are major cytokines of the innate immunity with main effects on the ensuing adaptive immune response (Seymour & Gemmell 2001). High IL-12 levels will contribute to the Th1 type immune reaction, as it is a strong inducer of IFN- γ production (Tsai et al. 2005). In contrast, IL-10 reduces the secretion of IFN- γ and has a potential role in diminishing IFN- γ -mediated responses and thereby Th1 type of immunity (Lappin et al. 2001). Furthermore, IL-1 is involved in the up-regulation of IFN- γ production by Th1 cells, down-regulation of IL-4 production by Th2 cells (Sandborg et al. 1995, Schmitz et al. 1993) and can be inhibited by smoking (Pabst et al. 1995). Taken together, the lower IL-12p40/IL-10 ratio and the lower IL-1 β production in the studied group of smokers were indicative of a stronger Th2 response (*Chapter 2*), which was confirmed by higher IL-13 levels produced by a Th2 cell population in smoking subjects (*Chapter 3*).

It is well known that there is a shift from a predominantly T cell to B cell lesion in the progression from gingivitis to periodontitis. It is interesting to speculate that a shift from cell-mediated immunity (Th1) to humoral immunity (Th2) occurs during the development of periodontal disease (Kinane & Lappin 2001). It is apparent that in gingivitis T cells probably exceed cells of the B cell lineage, and when this progresses into periodontitis, B cells then predominate (Kinane & Lappin 2001). The dominance of B-cells/plasma cells in the advanced/ progressive lesion would suggest a role for Th2 cells. Th1 and Th2 cells induce B cell proliferation, but Th2 cells are generally more efficient than Th1 in this capacity (Rothenmel et al. 1991). Furthermore, activated B cells have the potential to contribute to the amplification or maintenance of the ongoing polarized immune response (Harris et al. 2000). Therefore, it is plausible that a more pronounced Th2 response in smoking subjects may increase the B cell proliferation, inducing a stronger humoral response, which in turn may reinforce the existing Th2 response, thereby increasing the risk for recurrent periodontal destruction in the smoking patient.

B cells have the capacity to make pleiotropic cytokines such as IL-6 and tumor necrosis factor (TNF)- α , which regulate diverse aspects of bone resorption and formation in inflammatory diseases (Harris et al. 2000). These cytokines may induce bone

resorption directly and indirectly by affecting the production of the essential osteoclast differentiation factors (Boyce et al. 2005, Gemmell et al. 1997). Therefore, it may be suggested that these bone resorbing cytokines produced by increased activated B cells may contribute to the periodontal breakdown in the smokers. In addition, the pronounced B cell stimulation and activation may contribute to higher production of autoantibodies. It is known that autoimmune mechanisms may contribute to periodontal disease pathogenesis (Rajapakse & Dolby 2004). A recent study suggested the involvement of serum autoantibodies directed to extracellular matrix components in the pathogenesis of chronic periodontitis (De-Gennaro et al. 2006). In addition, a local production of autoantibodies to autoantigen in granulomatous tissues housed within the periodontal lesion has been described (Rajapakse & Dolby 2004). Therefore, autoimmune mechanisms triggered by the increase B cell activation may contribute to the periodontal breakdown in the smoking subjects. The previous studies on the influence of smoking on aspects of the immune response in the periodontal disease provided important background to further study the severity of periodontal breakdown observed in the periodontal patient. The aim of these studies was to provide a useful framework for the data analysis of our twin population.

In order to determine the relative contribution of genetic, environmental and life style factors; such as smoking, in the etiology of moderate to severe chronic periodontitis, the classic twin method was used. In *Chapter 4*, monozygotic (MZ) and dizygotic (DZ) twins reared together, were recruited to assess the contribution of genetics, periodontal pathogens and life style factors to the clinical phenotype. Important for this study was that the adult twin pairs were selected on the basis of at least one of the 2 twin members having interproximal attachment loss ≥ 5 mm in ≥ 2 non-adjacent teeth. The study included 10 MZ and 8 DZ twin pairs, in which the periodontal condition, presence of periodontal pathogens, educational level, smoking behavior and Body Mass Index (BMI) was evaluated. The most important result of this study is the finding that MZ twins appeared to be rather discordant with regard to mean attachment loss, number and percentage of teeth with AL ≥ 5 mm and percentage of teeth with bone loss $\geq 30\%$. By selection the MZ probands suffered from moderate to severe periodontitis, whereas the MZ co-twins were not selected on the presence of periodontitis and showed periodontal

breakdown to a much lesser extent. This finding was surprising since on the basis of previous research it was supposed that genetics contribute to 50% of the severity of periodontitis (Michalowicz et al. 2000) and thus less large differences were to be expected in the MZ twins. Analysis of our twin data showed that the lack of concordance could not be explained by periodontal bacteria, smoking and BMI, factors that are all known to be related to destructive periodontal disease. However, it must be realized that the number of twins in the present study was small. This small number may have been also responsible for the many non significant differences, e.g. the subgingival presence of *P. gingivalis*. In the MZ twin group, 5 out of the 10 probands were positive for *P. gingivalis*, whereas 2 out of the 10 co-twins were positive for this bacterium. A larger study population of MZ twins could have shown that *P. gingivalis* plays a significant role in the etiology of periodontitis. Smoking as an explanatory variable could not be evaluated in the MZ twins since these genetically identical pairs showed also a similar smoking behaviour. Interestingly, this was in contrast to smoking behaviour found within the DZ twins where no equal smoking habits between pairs were noted. In the DZ twins 45.6% of the variation in terms of periodontal breakdown could be explained by smoking. Within MZ twin pairs the discordance regarding periodontal breakdown was smaller than within DZ twin. This was expected as this phenomenon has been previously shown in large-scale twin data.

Since immune response mechanisms have been shown to play an important role in the periodontal breakdown, the analysis of the cytokine production in this twin population constituted an interesting parameter to be explored as a possible explanation for the discrepancy in periodontal phenotype between the MZ and DZ twin pairs. In *Chapter 5*, we investigated the extent of concordance in number of leukocytes and their cytokine secretion after *ex vivo* stimulation in the previously studied twin population. Probands of both MZ and DZ twin sets pooled together showed higher total numbers of leukocytes and lower IL-12p40 levels compared to their co-twins. The higher number of leukocytes is similar to findings in the literature where it has been shown that with increasing severity of periodontal disease, leukocyte numbers increase (Loos 2005). It is well known that lower IL-12p40 level favors the Th2 immunity and decreases the stimulation of the Th1 type immune response. Our finding of increased alveolar bone loss

together with the lower IL-12p40 production in the total group of probands compared to the group of co-twins is consistent with the characteristic Th2 type response in periodontitis. Furthermore, MZ probands secreted more IL-6 than their co-twins. This observation is in line with previous results showing a dose response in systemic levels of IL-6: increased severity leads to higher IL-6 secretion (Loos et al. 2000). IL-6 is a multifunctional cytokine, of which biological activities include B-lymphocyte differentiation, T-lymphocyte proliferation and stimulation of immunoglobulin secretion by B-lymphocytes (Hirano et al. 1990). Of particular significance is the ability of IL-6 to induce bone resorption, both by itself and in conjunction with other bone-resorbing agents (Ishimi et al. 1990). Therefore, it may be concluded that within our twin population, the higher IL-6 production in MZ probands seems to be associated to the increased bone loss found in this group. The results of our twin study suggested that the observed lack of strong concordance of periodontal destruction in the studied MZ twin population can not be explained by differences in numbers of leukocytes, but that low levels of IL-12p40 and high levels of IL-6 secretion after *ex vivo* stimulation may be regarded as risk indicators for the severity of periodontitis.

Based on our results from the twin population, we suggest that the role of genetics in periodontitis may have been overestimated. The host genetic make-up, the traditional lifestyle factors and the immune responses after stimulation of cells could not explain the lack of straight forward concordance in periodontal destruction, especially in MZ twins, indicative that other factors play an important role in the extent and severity of periodontal disease. We have several suggestions. For example, nutritional factors and dietary supplementation have been associated with the inflammatory response and disease severity in periodontal disease (Amaliya et al. 2007, Rosenstein et al. 2003, Staudte et al. 2005). A reduced-calorie diet dampens the inflammatory response and reduces active periodontal breakdown associated with an acute microbial challenge (Branch-Mays et al. 2008). In addition, fish oil dietary supplementation may have potential benefits as a host modulatory agent in the prevention and/or adjunctive management of periodontitis (Bendyk et al. 2009). Another explanation for discordance of periodontal breakdown among twins could be diversity of concomitant infectious agents in the periodontal tissues. Herpesvirus infections may initiate or accelerate

periodontal breakdown via their ability to stimulate cytokine release from host cells. The ensuing inflammation might impair host defense mechanisms, resulting in less defensive capacities against the resident periodontopathic bacteria (Contreras et al. 2000, Slots 2007).

Furthermore, although genetics modulate the inflammatory response, there is increasing evidence that epigenetic mechanisms are critical for regulating the inflammatory response (Offenbacher et al. 2008). Epigenetic events act through the remodeling of the chromatin structure due to DNA methylation and histone acetylation which can selectively activate or inactivate genes and determine their expression. In general, increased DNA methylation in the promoter region of genes, causes gene silencing (Franco et al. 2008). The epigenetic process, by inducing a change in cytokine profile, may subsequently influence the pathogenesis and determine the outcome of many infectious diseases (Gomez et al. 2009). For example, preliminary findings suggested that the gene for IL-6, a cytokine involved in the final differentiation of B-cells into immunoglobulin-secreting cells, undergoes a decrease in methylation in periodontal disease tissues compared to control samples. These preliminary findings suggest that the IL-6 gene may be preferentially upregulated in expression in periodontal disease (unpublished data, Offenbacher et al. 2008). This suggestion is in line with the finding in our twin study that in stimulated whole blood cell cultures, MZ probands suffering from moderate to severe periodontitis secreted higher levels of IL-6 than their co-twins with minor periodontal breakdown. It has been shown also that infection can lead to host epigenetic modification of an imprinted gene (Bobetsis et al. 2007). Thus previous infectious diseases, and/or viral infectious in the proband of a MZ twin pair could have triggered genetic variation and as a consequence developing more severe periodontal disease. Unfortunately, there is scarce information of epigenetic events during periodontitis. Hopefully, future research will help us understand, for example, how systemic exposures, like smoking, may alter global epigenetic patterns to affect the expression of periodontitis (Barros & Offenbacher 2009). Taken together, the above mentioned suggestions may potentially influence the disease expression and therefore could explain the lack of clear concordance in periodontal condition of the MZ twins in the present study.

The identification of individuals with increased susceptibility to periodontitis remains a great challenge in the dental practice. Tailored prevention and treatment strategies of the subject at higher risk for periodontitis are needed. Hopefully, studies on larger twin populations involving the assessment of other potential factors which may influence the host immune response may shed light on the pathways by which some individuals develop periodontitis and will provide knowledge on how to prevent effectively the disease onset and progression. It can be envisaged that in periodontitis, the interaction between genetic and epigenetic mechanisms influenced by life style- and environmental factors provides an interesting line of research in our field.